PERIODICUM BIOLOGORUM
VOL. 126, No 3–4, 159–176, 2024
DOI: 10.18054/pb.v126i3-4.32818

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

Original research article

Antagonistic effect of unionized ammonia (UIA) and *Aeromonas caviae* on the hemato-biochemical and histological responses of *Clarias magur*

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Keywords: *Aeromonas caviae*; *Clarias magur*; survival; sublethal; unionized ammonia

Received September 16, 2024
Revised March 21, 2025
Accepted March 24, 2025

Abstract

Background and purpose: Clarias magur *(Hamilton, 1822), the Asian or walking catfish, is a high-value fish species in India and Southeast Asia for its taste and nutritional value. The combined effect of ammonia and bacterial infection on* C. magur *has not been studied so far. Moreover, the mechanism of ammonia-induced toxicity and their adaptability in* C. magur *is poorly understood. Given this context, a systematic study was devised to examine the individual and combined exposure effect of unionized ammonia and* Aeromonas caviae *on haemato-biochemical and histopathological changes of different tissues of* C. magur.

Materials and Methods: *A 14-day experiment was conducted to expose the fish to sublethal doses of UIA,* A. caviae*, and a combination of both. Initially, we have determined the 96-hr* LC_{50} *value for UIA in* C. magur. *During the experimental period, various hematological parameters were tested, such as total erythrocyte count, hemoglobin, packed cell volume, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration. Different biochemical parameters like blood glucose, serum, and tissue urea, serum glutamate pyruvate transaminase, and serum glutamate oxaloacetate transaminase levels and histopathological examination were performed.*

Results: *The 96-hr* LC_{50} *value for UIA in* C. magur *was 7.21* mgL^{-1}. *The* 14^{th} *day exposure of UIA (2.4* mgL^{-1}*),* A. caviae *(*1.53×10^5 *CFU* mL^{-1}*), and their combination resulted in significant decreases in various hematological parameters such as total erythrocyte count, hemoglobin, packed cell volume, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration. Additionally, there were notable increases in blood glucose, serum and tissue urea, serum glutamate pyruvate transaminase, and serum glutamate oxaloacetate transaminase levels in all treatment groups compared to the control. Histological examination revealed significant changes in gill, kidney, and liver tissues, including lamellar fusion, edema, nodular enlargement of lamellar tips, lamellar congestion and curling in gill tissues, cytoplasmic vacuolation, pyknosis, karyorrhexis, karyolysis, and degeneration of renal tubule cells in kidney tissues, and increased vacuolation in hepatocytes, disorganization of hepatic cords, and increased hemorrhage in liver tissues. Interestingly, the combined exposure to UIA and* A. caviae *resulted in fewer histological alterations than individual exposures. Furthermore, after the* 14^{th} *day, the group exposed only to bacteria exhibited the lowest relative percent survival compared to those exposed to only UIA and the combination.*

Conclusions: *This study signifies that there was a negative synergistic or antagonism effect in the fish after exposure to ammonia and bacteria in combination. This discrepancy indicates that elevated ammonia concentrations might have restricted the survival of* A. caviae *or disrupted the infection process. The present study's findings open a new avenue for further understanding the mechanism of ammonia exposure in the direction of an alternative method to combat bacterial infections.*

INTRODUCTION

The aquaculture industry faces considerable setbacks due to disease outbreaks, with global losses estimated at approximately $6 billion each year and these losses stem from higher mortality rates and overall declines in animal health and productivity *(1)*. A significant factor contributing to widespread of fish mortality is the interaction of three main elements: toxic substances or pollutants, environmental conditions, and disease-causing pathogens *(2)*. Unlike terrestrial environments, the aquatic environment provides a favorable breeding ground for pathogenic bacteria, irrespective of their host species *(3)*. Changes in water chemistry can directly harm fish, while even sub-lethal changes may cause stress, rendering more susceptible to infections *(4)*. *Clarias magur* (Hamilton, 1822), also known as the Asian or walking catfish, is a high value fish species in India and Southeast Asia for its taste and nutritional value. Despite its commercial significance, it is now classified as 'endangered' on the IUCN Red List due to its significant decline in the wild population caused by various known and unknown factors, including environmental parameters and potentially harmful diseases *(5)*. This catfish, typically found in swampy water environments, used to face challenges such as hyper-ammonia and desiccation stresses that result in increased ammonia accumulation and toxicity. However, the specific mechanisms underlying this ammonia toxicity remain unclear *(6, 7)*.

Ammonia is the primary nitrogenous compound excreted by fish which can significantly impact fish productivity and enters natural water system through various pathways. The concentration of ammonia can escalate swiftly in confined water bodies and intensive fish farming systems due to the continuous buildup of feed wastes *(8)*. Fishes naturally generate ammonia during the breakdown of organic substances like protein, releasing it primarily through gill membranes and to a lesser extent through urine *(9)*. Additionally, anthropogenic activities like sewage discharge, industrial waste, and agricultural runoff containing chemical fertilizers can also elevate ammonia levels in aquatic environments *(6)*. In aquaculture systems, ammonia exists in equilibrium between ionized (NH_4^+) and unionized (NH_3) forms, with the latter being more toxic as it can easily penetrate fish gills and convert to NH_4^+ internally to establish an equilibrium between internal and external un-ionized ammonia concentrations, leading to cellular damage *(10)*.

Ammonia impedes oxygen transfer from gills to blood, resulting in immediate and prolonged gill damage. Fish affected by ammonia poisoning often exhibit sluggish behavior, appearing near the water surface and seemingly gasping for air *(11)*. Ammonia can be acutely toxic to fish mainly due to its effects on the central nervous system, leading to "acute ammonia intoxication," which may include convulsions and death *(12)*.

Pathogens consistently release virulence factors as their strategy to evade host defenses, ultimately aiding in the establishment of infections *(13)*. Magur catfish are susceptible to bacterial and fungal infections throughout their farming cycle. Several Gram-negative bacterial pathogens have been identified in magur, including *Aeromonas hydrophila, Aeromonas veronii, Aeromonas caviae, Flavobacterium spp, Edwardsiella tarda, Edwardsiella ictaluri,* and *Pseudomonas spp (14, 15)*. Most species belonging to the *Aeromonas* genus are pathogenic to *C. magur* including *Aeromonas caviae*. Recently *A. caviae* infection has been found to cause up to 70% mortality in the hatchery and grow-out culture of magur fingerlings *(16)*.

Aeromonads are commonly present in freshwater cultivation systems, and the rapid increase in ammonia level within the aquatic system poses a significant constraint in aquaculture production. The coexistence of these two factors can have a substantial impact on the health of fish. In some studies, it has been found that ammonia toxicity has increased the disease susceptibility of fish *(17)*, whereas, in some cases there has been found to decrease the susceptibility *(18)*. A decrease in protection against bacteria (*Streptococcus iniae*) in ammonia-exposed trout was reported by Hurvitz *et al. (18)*. Whereas there seemed to be no association between the concentration of unionized ammonia and in the mortality of tilapia fingerling as reported by Zainal *et al. (19)*. In contrast, ammonia limited the *Flavobacterium columnare* infection in channel catfish by interfering with the infection process thus causing an increase in protection *(17)*. The Combined effect of ammonia and bacterial infection together on *Clarias magur* has not been studied so far. Moreover, the mechanism of ammonia induced toxicity and their adaptability in *C. magur* is poorly understood. Given this context, a systematic study was devised to examine the individual and combined exposure effect of un-ionized ammonia and *Aeromonas caviae* on haemato-biochemical and histopathological changes of different tissues of *C. magur*.

MATERIALS AND METHODS

Collection and maintenance of the experimental fish

C. magur juveniles (length 12 ± 1.0 cm and weight 45 ± 2.0 g) were procured from a certified Magur hatchery in West Bengal, India and transported to the wet lab at ICAR-CIFE, Mumbai, in airtight polythene bags with

oxygen (100 fish per pack). Three hundred fish were used for the acute toxicity study and 100 for the combined toxicity study. Upon arrival, fish underwent a dip treatment with potassium permanganate (5 mg L^{-1}) as a precaution and after that they were transferred to 200 L capacity plastic tank (20 fish in each tank) and being acclimatized in the wet laboratory complex of ICAR-CIFE for 21 days. Adequate aeration was provided, and fish were fed with extruded floating pellets containing 32% protein twice a day @ 3% body weight (w/w). Three hundred fish were used for the acute toxicity study and 100 for the combined toxicity study. For toxicity tests, fish were placed in rectangular plastic crates (62 L capacity) with bore well water. The tanks were disinfected with alcohol, treated with $KMnO_4$ (4 mg/L), and rinsed. Water volume was maintained at 22 L for acute toxicity and 30 L for chronic toxicity studies. The handling of this particular endangered fish species has been performed according to the ethical guidelines of the institutional ethical committee of ICAR-CIFE, Mumbai.

Preparation of the toxicant stock solution and standard curve of ammonia

The test solution was made using ammonium chloride (NH_4Cl) sourced from SRL, India, having a concentration of 33.37 gm of ammonium (NH_4^+) per 100 gm of NH_4Cl. The samples of known concentration of total ammonia nitrogen (TAN) were analyzed using the phenate method of Eaton *et al. (20)* and a standard curve was prepared against the obtained values. The concentration of unionized ammonia was estimated based on water pH and temperature, following the method outlined by Emerson *et al. (21)*.

Bacterial strain and plotting of bacterial growth curve

A virulent strain (BLBM-05) (Accession no – MT973994.1) of *Aeromonas caviae*, obtained from ICAR-CIFE, Mumbai, was maintained in the Aquatic Animal Health Management Division. A growth curve for *A. caviae* was prepared by measuring the optical density (OD) of the bacterial broth culture in Brain Heart Infusion (BHI) broth at 600 nm at various time intervals over a period of 96 h.

Experimental design

Determination of median lethal concentration of unionized ammonia in *Clarias magur*

Fish acute toxicity experiment had been carried out following the Organization for Economic Co-Operation and Development (OECD) guideline no 203 *(22)*. Bioassay experiments were conducted in 50-liter plastic crates filled with 30 liters of tap water. To determine the median lethal concentration of UIA in *Clarias magur* a 96-hr range finding test was conducted. This test was performed following the standard method outlined in APHA *(23)* and involved administering five different concentrations of UIA (with triplicates for each treatment group): 0, 20, 40, 60, 80 and 100 mgL^{-1} of UIA at a water pH of 8.1±0.2 and a temperature of 30±0.5°C.

Based on the range finding test result, the short-term (96 h) definitive acute toxicity test was designed and performed taking seven different test concentrations, i.e. 0 mgL^{-1} (0 mgL^{-1} of TAN), 3 mgL^{-1} (36 mgL^{-1} of TAN), 6 mgL^{-1} (72 mgL^{-1} of TAN), 9 mgL^{-1} (108 mgL^{-1} of TAN), 12 mgL^{-1} (142 mgL^{-1} of TAN), and 15 mgL^{-1} (178 mgL^{-1} of TAN) and 18 mgL^{-1} (214 mgL^{-1} of TAN) unionized ammonia (UIA), along with a control. The dose-response curve of the test organism was determined by plotting probit transformed percent mortality against log concentration *(24)*.

Sub-lethal toxicity test

There after based on the obtained median lethal concentration of UIA in *C. magur* the sub-lethal toxicity test was planned for a period of 14 days *(25)*. The study comprised of three subsets: the first subset received a concentration of 2.4 mgL^{-1} (one-third of the LC_{50} value) of ammonia treatment, the second subset was exposed to 1.53 x 10^5 CFU mL^{-1} (one-tenth of the LD_{50} value) *(16)* concentration of bacterial infection, and the third subset experienced the combined application of both ammonia and bacteria to evaluate combined effect in triplicate. Sampling occurred on the 3rd, 7th, and 14th day of the experiment.

Throughout the experimental period, various water quality indicators were carefully tested in accordance with the guidelines outlined by APHA *(23)*. The parameters assessed included temperature, pH level, dissolved oxygen content, alkalinity, hardness, total ammonia nitrogen and unionized ammonia.

Hemato-biochemical analysis

Fish were anesthetized with eugenol (4-allyl2-methoxyphenol) before blood collection and 1 mL tuberculin syringe rinsed with a 2% EDTA solution was used for collecting the blood samples. Blood samples were divided into EDTA-containing vials for immediate hematological analysis and vials without EDTA for serum collection. Serum samples were obtained by centrifugation at 3500 rpm for 5 min at 4°C and stored at –20°C until use.

Standard methods were employed to evaluate hematological indices. The Total Erythrocyte Count (TEC) or Red Blood Cell (RBC) count was performed following Schaperclaus *et al. (26)*. Blood was drawn into an RBC pipette up to the 0.5 mark, then Hayem's solution (Qualigens, India) was added to the 101 mark (1:200 dilution). The mixture was rotated for 1 minute and a small drop was introduced into the counting chamber via capillary action. After 3 minutes for cell settling, erythrocytes were counted using a trinocular microscope at 40X magnifica-

tion across 5 group squares, and the TEC was calculated using a standard formula:

$$\mathrm{TEC}\left(\mathrm{mm}^{-3}\right)=\frac{\text{No. of cells}\times\text{dilution factor}\times\text{total area}}{\text{Area count}}$$

Where, dilution factor = 200, depth factor = 10, total ruled area = 25, area count = 5.

The White Blood Cell (WBC) or Total Leucocyte Count (TLC) was conducted following Schaperclaus *et al.* (26). Blood was drawn into a WBC pipette up to the 0.5 mark, then WBC diluting fluid (Himedia) was added to the 11 mark (1:20 dilution). The mixture was gently rotated for 1 minute. A small drop of diluted blood was introduced into the counting chamber by capillary action and left for 3 minutes to allow the cells to settle. WBCs were counted in the large squares at the four corners, with cells on two adjacent margins included and the others discarded.

$$\mathrm{TLC}\left(\mathrm{mm}^{-3}\right)=\frac{\text{No. of cells}\times\text{dilution factor}\times\text{depth factor}}{\text{Area count}}$$

The Packed Cell Volume (PCV) or hematocrit (Hct) was measured according to Schaperclaus *et al. (26)*. Blood was drawn into a heparinized capillary tube up to the 100 mark, sealed with wax, and centrifuged at 3000 rpm for 3 minutes. After centrifuging, the capillary tube was placed on a reading device, and the volume was recorded. The PCV or hematocrit value was expressed as the percentage fraction of whole blood cells (volume %).

Hemoglobin was estimated by Drabkin's cyanmethemoglobin method *(27)*. The technique employed standardized hemoglobin pipettes. Twenty milliliters of blood were added to 5 mL of Drabkin's solution, thoroughly mixed, and set aside for 5 min. In the spectrophotometer, readings were taken at 530 nm. The hemoglobin values were calculated using the hemoglobin standard and a hemoglobin curve.

MCV expressed as femtoliters (10–15 L) was calculated using the following formula *(27)*:

$$\mathrm{MCV}\left(\mathrm{fL}\right)=\frac{\text{Packed cell volume}\left(\%\right)}{\text{RBC count}\left(\text{EC in a million per cubic mm}\right)}\times 10$$

The average hemoglobin mass per erythrocyte in a blood sample is known as mean corpuscular hemoglobin (MCH). The absolute amount of hemoglobin in an average red blood cell in a sample was represented by this value. The MCH was calculated from the Hb and RBC (28):

$$\mathrm{MCH}\left(\mathrm{pg}\right)=\frac{\text{Hemoglobin}\left(\mathrm{g/dL}\right)}{\text{RBC Count}\ \left(\text{EC in a million per cubic mm}\right)}\times 100$$

MCHC was calculated using the following formula: grams of hemoglobin per 100 mL packed cells *(27)*:

$$\mathrm{MCHC}\left(\%\right)=\frac{\text{Hemoglobin}\left(\mathrm{g/dL}\right)}{\text{Packed cell volume}\left(\%\right)}\times 100$$

Blood glucose, serum total protein, serum albumin and globulin, serum glutamic-oxaloacetic transaminase (SGOT), Serum glutamic pyruvic transaminase (SGPT), urea (serum and tissue) of different experimental fish groups were measured spectrophotometrically as per the diagnostic protocol of Erba kit (Germany).

Histopathology of gill, kidney and liver tissues

Histopathological observation was carried out following the standardized method outlined in Roberts *(29)*. Gill, liver, and kidney tissues were promptly collected and fixed in NBF solution. After a series of steps, including fixation, dehydration, embedding, sectioning, and staining, Inspection of the prepared slides of gill, liver, and kidney tissues was conducted under the Leica DM 750 light microscope at ×10, ×40 and ×100 magnifications.

Relative percentage survival

The Relative percentage survival (RPS) was calculated by following formula (30) as given below:

$$RPS=\left(\frac{\text{Number of surviving fish after exposure}}{\text{Number of fish being exposed}}\right)\times 100$$

Statistical analysis

Statistical software, IBM SPSS Statistics for Windows, Version 22.0 (Released 2013. IBM Corp., Armonk, NY), was used to analyze all the data. All the data were expressed mean ± SE of three replicates (n=3). The data were subjected to one-way ANOVA and Duncan post hoc for significant differences at a p value of $p < 0.05$.

RESULTS

LC_{50} value of unionized ammonia in Clarias magur

The standard curve obtained from different graded concentration of total ammonia nitrogen has been depicted in Figure 1 yielding an R-squared value of 0.998.

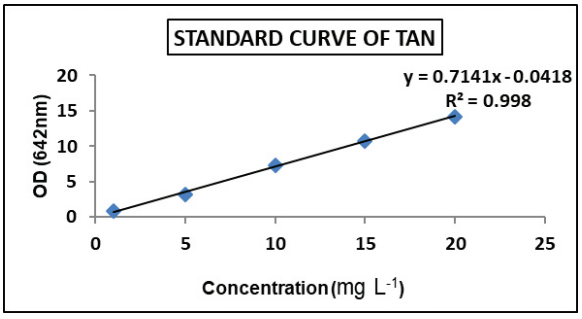

Figure 1. *Standard curve of TAN (total ammonia nitrogen) of different graded concentrations.*

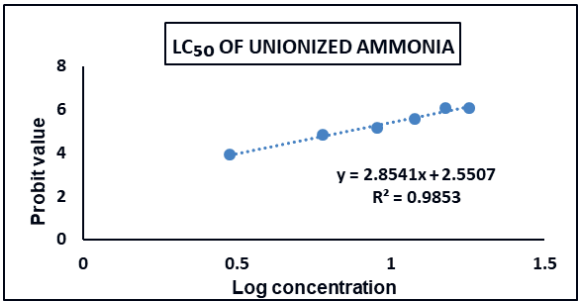

Figure 2. *LC_{50} value of UIA (Unionized ammonia) in Clarias magur by exposing fishes to different concentrations of unionized ammonia (UIA) viz. 0, 3, 6, 9, 12, 15 and 18 mg L^{-1}.*

The 96-hr LC_{50} value of unionized ammonia in *C. magur* was found to be 7.21 mgL^{-1} (Figure 2).

Bacterial growth curve

Upon plotting the growth curve for *Aeromonas caviae*, it was observed that the bacterial growth entered the exponential phase between 12 and 24 hr (Figure 3).

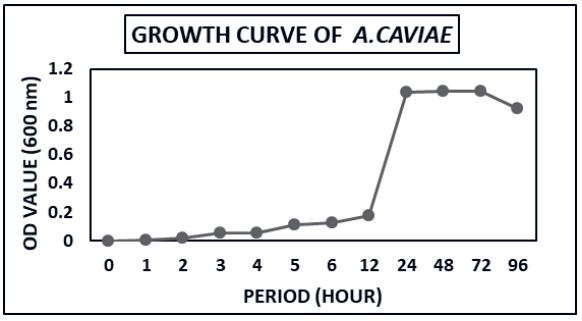

Figure 3. *Growth curve of Aeromonas caviae over a period of 96 hr.*

Physico-chemical parameters of water

Physico-chemical parameters of water were recorded (Table 1) pre and during 14-day experimental period of unionized ammonia in *C. magur*. The pH, DO, temp, ammonia, hardness, and alkalinity levels in all the conditions were recorded in the optimum range for rearing *C. magur*.

Hematological responses

TEC, TLC, Hb, PCV, MCV, MCH, and MCHC concentration of *C. magur* exposed to different concentrations of sub-lethal UIA, bacteria and both ammonia and bacteria have been presented in Figures 4A–G. A significant ($p < 0.05$) decrease in the hematological parameters was observed in all the treatment groups as compared to the control throughout the 14-day exposure period. On the 14th day of experiment maximum reduction in TEC ($1.198\pm0.1\times10^6$ uL^{-1}) and PCV (22.8 ± 0.2 uL^{-1}) was ob-

Table 1. *Physico-chemical parameters of water were recorded during the experimental period.*

Parameter	Control			Ammonia			Bacteria			Ammonia and bacteria		
	3rd	7th	14th	3rd	7th	14th	3rd	7th	14th	3rd	7th	14th
pH	8.17±0.09	8.07±0.07	7.97±0.29	7.86±0.16	7.96±0.20	8.16±0.11	8.19±0.06	8.09±0.10	8.29±0.05	7.71±0.03	7.81±0.02	8.01±0.04
Temperature (°C)	31.21±0.51	31.51±0.58	31.21±0.58	31.0±0.25	31.55±0.24	32.0±0.35	32.47±0.27	31.47±0.22	32.47±0.29	30.89±0.55	31.89±0.60	32.89±0.15
Hardness (mgL–1)	183.79±0.59	180.79±0.59	175.79±0.59	187.10±0.44	189.10±0.49	183.10±0.94	187.69±0.84	182.69±0.89	179.69±0.83	188.90±0.33	186.90±0.14	189.90±0.93
Alkalinity (mgL–1)	132.25±1.36	130.25±1.36	135.25±1.36	147.14±1.18	144.14±1.11	140.14±1.11	133.54±1.23	130.54±1.23	139.54±1.23	142.67±0.52	147.67±0.32	143.67±0.59
DO (mgL–1)	5.03±0.09	5.03±0.04	4.93±0.09	4.77±0.09	4.70±0.06	4.87±0.10	4.87±0.07	4.73±0.09	5.17±0.17	4.73±0.08	4.63±0.18	4.63±0.38
TAN (mgL–1)	0.21±0.04	0.31±0.09	0.41±0.01	40.79±0.23	39.79±0.28	42.79±0.93	0.25±0.03	0.45±0.05	0.29±0.09	41.56±0.76	40.56±0.75	41.56±0.46
UIA (mgL–1)	0.06±0.02	0.06±0.03	0.06±0.05	2.44±0.04	2.24±0.04	2.34±0.10	0.25±0.03	0.35±0.01	0.25±0.01	2.50±0.05	2.40±0.15	2.60±0.09

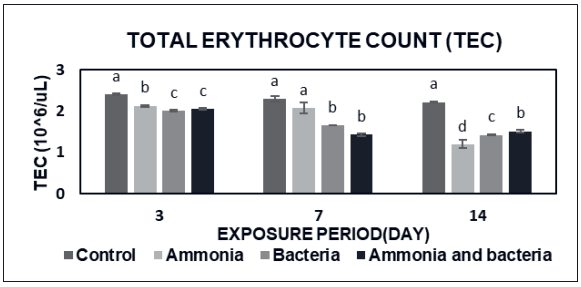

Figure 4A. *Effect of ammonia, bacteria and combined (ammonia and bacteria) exposure on total erythrocyte count (TEC) (×10^6 mm^{-3}) of C. magur. Data is shown in mean ± SE of three replicates (n=3). Different letters indicate significant differences among the treatment groups within the same exposure period at levels of p < 0.05.*

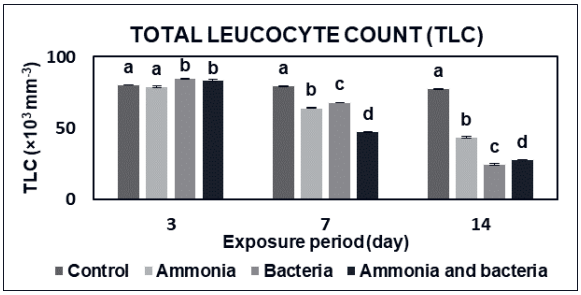

Figure 4B. *Effect of ammonia, bacteria and combined (ammonia and bacteria) exposure on total leucocyte count (TLC) (×10^3 mm^{-3}) of C. magur. Data is shown in mean ± SE of three replicates (n=3). Different letters indicate significant differences among the treatment groups within the same exposure period at levels of p < 0.05.*

served in the ammonia exposed group & maximum decrease in Hb and MCH was observed in the bacteria exposed fish group. Notably white blood cell (WBC) count reduced significantly (p<0.05) in ammonia exposed fish group (43.5± 0.34×10^3 µL^{-1}) as compared to the control group (80. 19± 0. 09×10^3 µL^{-1}) on the 3rd day of exposure

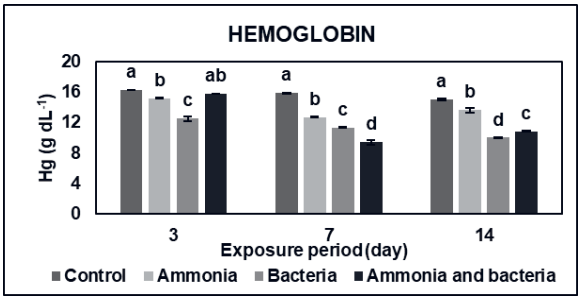

Figure 4C. *Effect of ammonia, bacteria and combined (ammonia and bacteria) exposure on hemoglobin content (g dL^{-1}) of C. magur. Data is shown in mean ± SE of three replicates (n=3). Different letters indicate significant differences among the treatment groups within the same exposure period at levels of p < 0.05.*

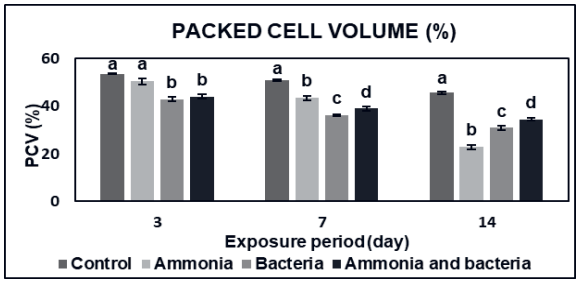

Figure 4D. *Effect of ammonia, bacteria and combined (ammonia and bacteria) exposure on packed cell volume (%) of C. magur. Data is shown in mean ± SE of three replicates (n=3). Different letters indicate significant differences among the treatment groups within the same exposure period at levels of p < 0.05.*

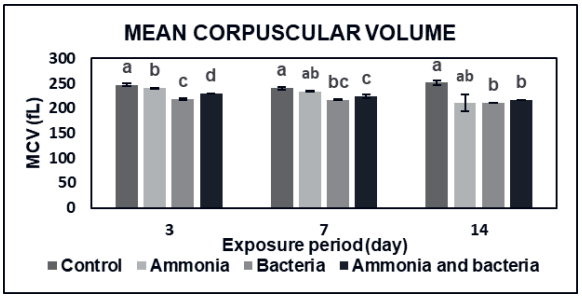

Figure 4E. *Effect of ammonia, bacteria and combined (ammonia and bacteria) exposure on mean corpuscular volume (MCV) (fL) of C. magur. Data is shown in mean ± SE of three replicates (n=3). Different letters indicate significant differences among the treatment groups within the same exposure period at levels of p < 0.05.*

and reduced significantly till the 14th day. But in the case of bacteria & combined exposed fish group there has been found a little increase in TLC initially on the 3rd day but a gradual decrease at the later stage. While in the case of MCV and MCHC values, no significant differences have been observed between the different treatment groups.

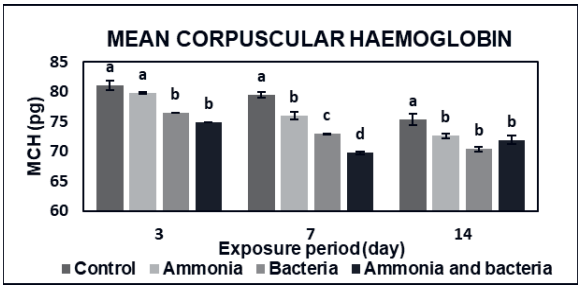

Figure 4F. *Effect of ammonia, bacteria and combined (ammonia and bacteria) exposure on mean corpuscular hemoglobin (MCH) (pg) of C. magur. Data is shown in mean ± SE of three replicates (n=3). Different letters indicate significant differences among the treatment groups within the same exposure period at levels of p < 0.05.*

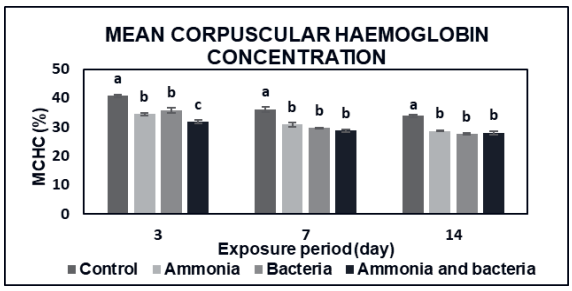

Figure 4G. *Effect of ammonia, bacteria and combined (ammonia and bacteria) exposure on mean corpuscular hemoglobin concentration (MCHC) (%) of C. magur. Data is shown in mean ± SE of three replicates (n=3). Different letters indicate significant differences among the treatment groups within the same exposure period at levels of p < 0.05.*

Biochemical parameters

Blood glucose, total protein, serum albumin, serum globulin, SGPT, SGOT, serum and tissue urea concentrations of *C. magur* exposed to individual and combined exposure effect of UIA and bacteria are presented in Figures 5A–H. During our experiment, serum blood glucose levels were observed to rise significantly ($p < 0.05$) across all treatments group, with the bacterial treatment group displaying the highest glucose content of 71.67±0.55 mgdL^{-1} on the 14th day of experiment as compared to the control group (20.7±0.15 mgdL^{-1}). Total protein, serum albumin, and globulin experienced a significant ($p < 0.05$) decline across all experimental groups in comparison with the control. Initially serum total protein level was found to increase in the bacteria and combined exposed fish group with a highest value of 1.8±0.008 gdL^{-1} in the combined exposed group. But at later stage it significantly ($p < 0.05$) got reduced in all the treatment groups with lowest value of 2.48±0.09 gdL^{-1} in the bacteria exposed group. Total albumin and globulin content got reduced gradually in all the treatment groups until the 14th day of experiment. Conversely, the activities of SGOT and SGPT in serum displayed a significant increase ($p < 0.05$) in all experimental groups compared to the control. Regarding urea concentration in both serum and tissue samples, the highest accumulation of 19.21±0.29 mgdL^{-1} urea in serum and 8.38±.17 mgdL^{-1} urea in tissue sample was observed in fish exposed to only ammonia.

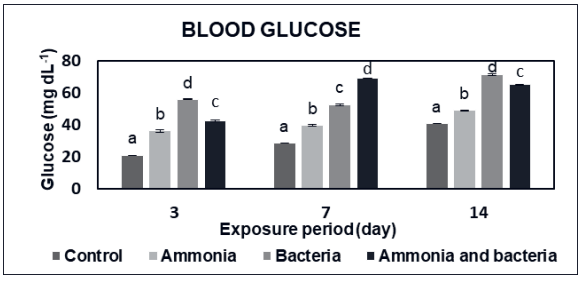

Figure 5A. *Effect of ammonia, bacteria and combined (ammonia and bacteria) exposure on blood glucose concentration (mg dL^{-1}) of C. magur. Data is shown in mean ± SE of three replicates (n=3). Different letters indicate significant differences among the treatment groups within the same exposure period at levels of p < 0.05.*

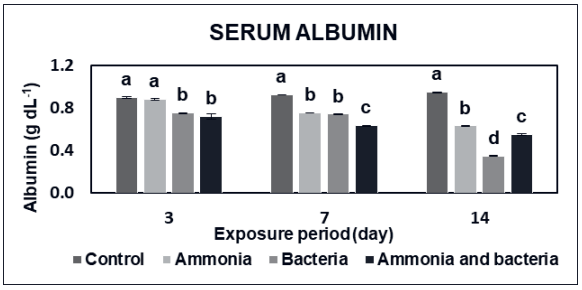

Figure 5C. *Effect of ammonia, bacteria and combined (ammonia and bacteria) exposure on serum albumin (g dL^{-1}) of C. magur. Data is shown in mean ± SE of three replicates (n=3). Different letters indicate significant differences among the treatment groups within the same exposure period at levels of p < 0.05.*

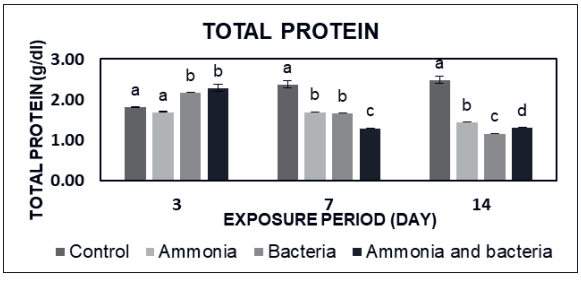

Figure 5B. *Effect of ammonia, bacteria and combined exposure (ammonia and bacteria) on total protein concentration (g dL^{-1}) of C. magur. Data is shown in mean ± SE of three replicates (n=3). Different letters indicate significant differences among the treatment groups within the same exposure period at levels of p < 0.05.*

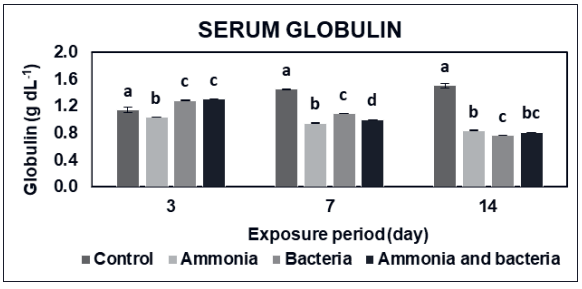

Figure 5D. *Effect of ammonia, bacteria and combined (ammonia and bacteria) exposure on serum globulin (g dL^{-1}) of C. magur. Data is shown in mean ± SE of three replicates (n=3). Different letters indicate significant differences among the treatment groups within the same exposure period at levels of p < 0.05.*

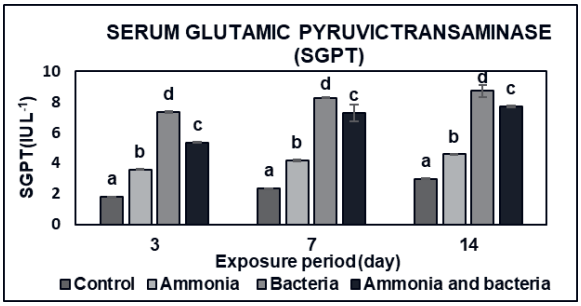

Figure 5E. *Effect of ammonia, bacteria and combined (ammonia and bacteria) exposure on serum glutamate pyruvate transaminase (IU L^{-1}) of C. magur. Data is shown in mean ± SE of three replicates (n=3). Different letters indicate significant differences among the treatment groups within the same exposure period at levels of p < 0.05.*

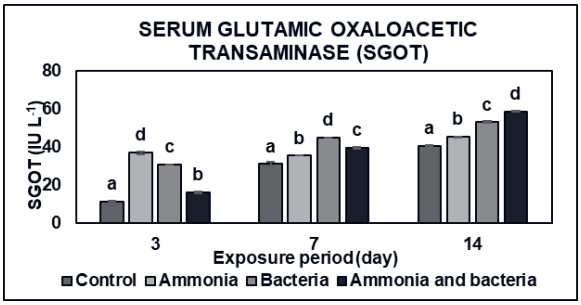

Figure 5F. *Effect of ammonia, bacteria and combined (ammonia and bacteria) exposure on serum glutamate oxaloacetate transaminase (IU L^{-1}) of C. magur. Data is shown in mean ± SE of three replicates (n=3). Different letters indicate significant differences among the treatment groups within the same exposure period at levels of p < 0.05.*

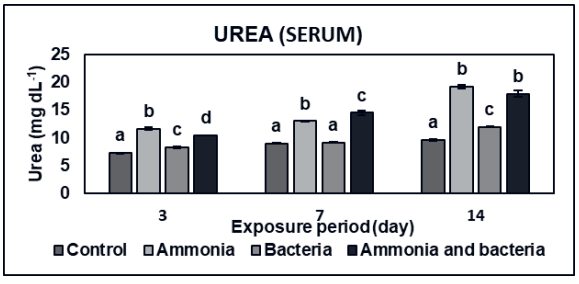

Figure 5G. *Effect of ammonia, bacteria and combined (ammonia and bacteria) exposure on serum urea (mg dL^{-1}) of C. magur. Data is shown in mean ± SE of three replicates (n=3). Different letters indicate significant differences among the treatment groups within the same exposure period at levels of p < 0.05.*

Relative percentage survival (RPS)

In our present study there has been found a significant (p<0.05) decrease in fish relative percent survivability as compared to control. In case of bacteria exposed fishes, the lowest survivability was observed (60%) then the

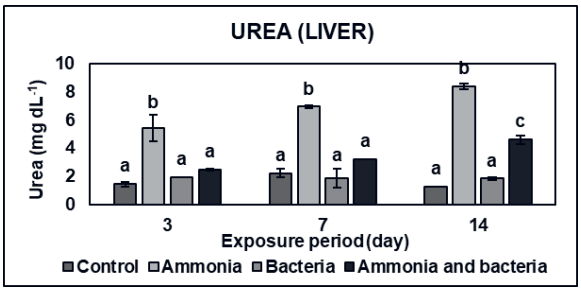

Figure 5H. *Effect of ammonia, bacteria and combined (ammonia and bacteria) exposure on liver tissue urea (mg dL^{-1}) of C. magur. Data is shown in mean ± SE of three replicates (n=3). Different letters indicate significant differences among the treatment groups within the same exposure period at levels of p < 0.05.*

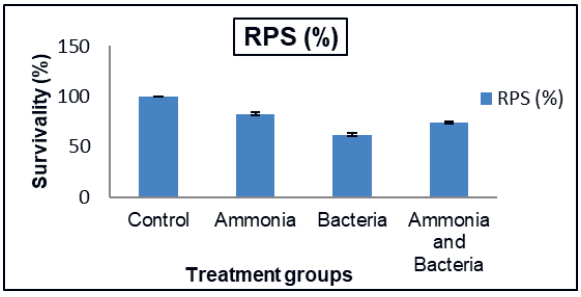

Figure 6. *Effect of ammonia, bacteria and combined (ammonia and bacteria) exposure on relative percentage survival (%) of C. magur. Data is shown in mean ± SE of three replicates (n=3).*

combined (75%) and ammonia exposed (60%) fish group as compared to the control (Figure 6).

Histopathological alteration in gill, kidney and liver tissue

Figures 7A–E, 8A–F, and 9A–F illustrate histopathological observations in fish gill tissues of control and treated groups. Initially exposure to ammonia alone leads to mild atrophy, epithelial shortening, and infiltration of immature erythrocytes and leucocytes in primary lamellae, progressing to lamellar hyperplasia, fusion, clubbing of lamellar tips, fluid accumulation, loss of lamellae and other degenerative changes at later stages. Bacterial exposure causes deformation, hyperplasia, and fusion of secondary lamellae, progressing to curling, sloughing, and increased cellularity. Combined exposure results in epithelial hyperplasia, lamellar fusion, and eventual necrosis and disruption of gill epithelium after prolonged exposure.

Figures 10A, B, 11A–C, and 12A–C depict histological changes in fish kidney tissues exposed solely to ammonia, solely bacteria, and both ammonia and bacteria. Ammonia exposure results in cellular pyknosis, nuclear

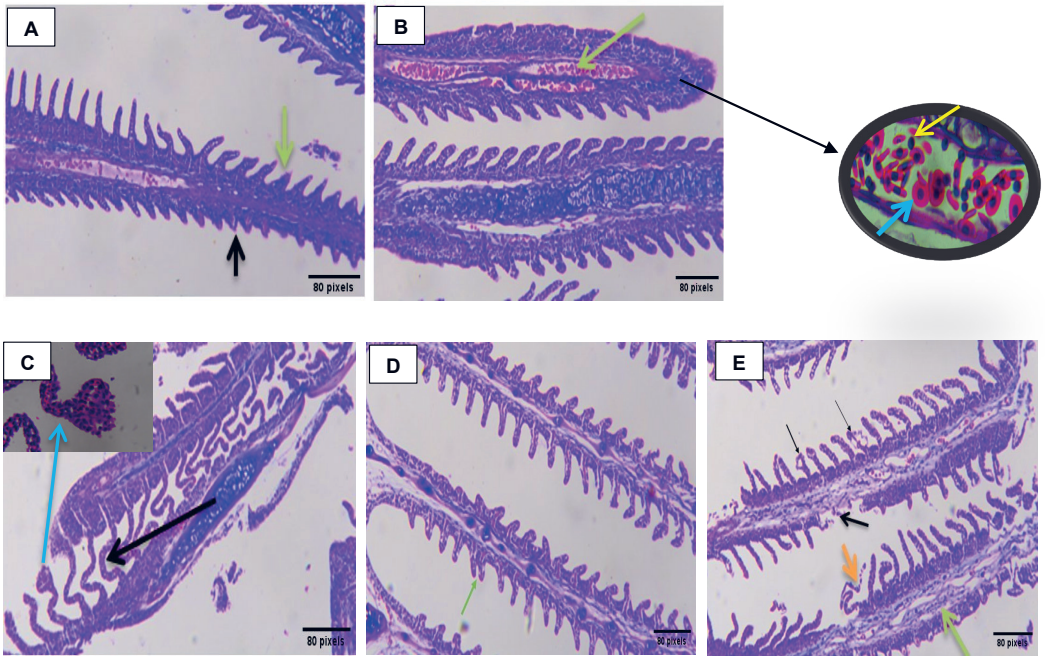

Figure 7. *Histological changes observed in gill tissue after 3rd day (A, B) and after 14th day (C, D, E) of only ammonia exposure. (A) Desquamation and necrosis (green arrow), mild atrophy and shortening in the epithelial lining of the secondary lamellae (black arrows) (H&E; X40). (B) Infiltration of erythrocyte and leukocyte (green arrow) (H&E; X40), Lymphocyte (yellow arrow), immature erythrocyte (blue arrow) (H&E; X100). (C) Clubbing the tips of secondary lamellae (thick black arrow) (H&E; X40), Hyperplasia (blue arrow) (H&E; X100). (D) Fusion of secondary lamellae (green arrow) (H&E; X40). (E) Degeneration and vacuolation of cartilaginous bar (green arrow), epithelial lifting and edema (thin arrow), deformed (irregular shape) secondary lamellae (thick black arrow), Sloughing of epithelial cells (SE) and loss of lamellae (L) (thick black arrow) (H&E; X40).*

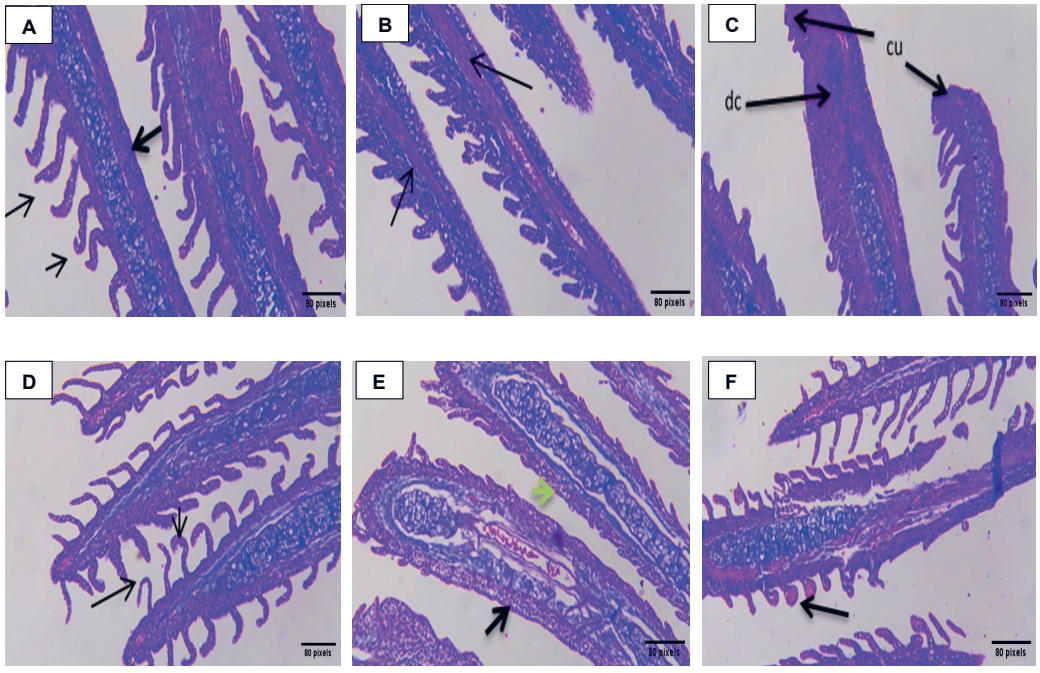

Figure 8. *Histological changes observed in gill tissue after 3rd day (A, B, C) and 14th day (D, E, F) of only bacterial exposure. (A) Deformed secondary lamellae (thin arrow), complete fusion of Secondary lamellae (thick arrow) (H&E; X40). (B) Congestion (thin arrow) (H&E; X40). (C) Gill tissues with curling of primary lamellae (cu) and distal clubbing (dc) of secondary lamellae in the gills (H&E; X40). (D) Curling of secondary lamellae (thin arrow) (H&E; X40). (E) Complete fusion of secondary Lamellae (black arrow), incomplete fusion of secondary lamellae (green arrow) (H&E; X40). (F) Nodular enlargements of the lamellar tips with increased internal cellularity (arrow) (H&E; X40).*

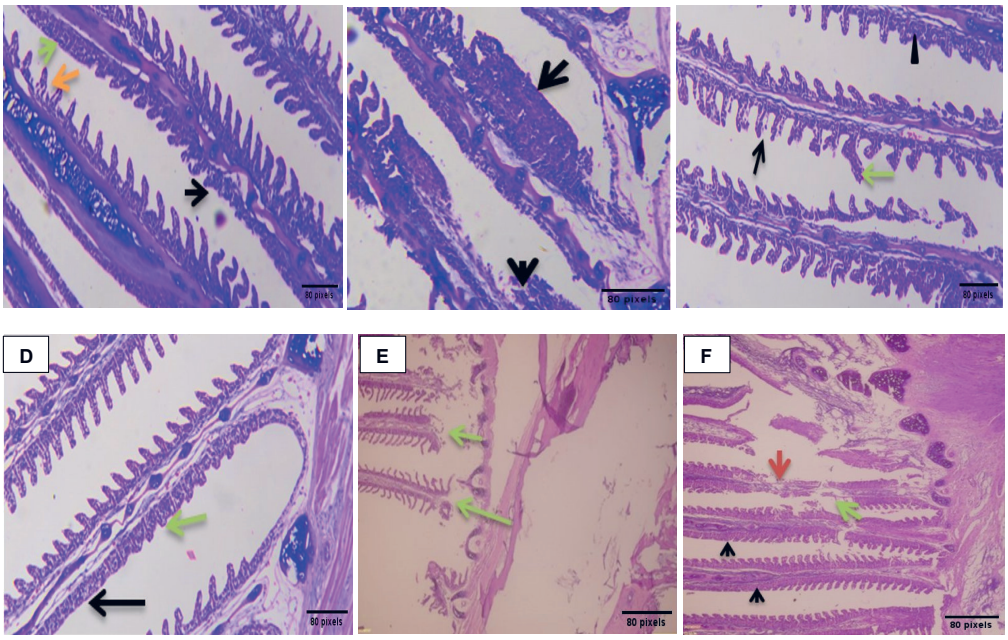

Figure 9. *Histological changes observed in gill tissue after 3rd day (A, B, C) and 14th day (D, E, F) of combined exposure (both ammonia and bacteria). (A) Epithelial lifting (orange arrow), complete fusion (green arrow) and in complete fusion (black arrow) of gill lamellae (H&E; X40). (B) Epithelial hyperplasia leading to complete lamellar fusion (arrow head) and necrosis (arrow) (H&E; X40). (C) Separation of epithelial surface on Lamellae (arrow), incomplete lamellar fusion (arrow head) & hyperplasia (H&E; X40). (D) Incomplete fusion (green arrow) and complete fusion (black arrow) of gill lamellae (H&E; X40). (E) Separation of gill filament from mucosal epithelium and basement membrane (arrow) (H&E; X40). F. Fusion of gill lamellae (arrow head), gill epithelium was disrupted owing to degeneration (green arrow) and necrosis (red arrow) (H&E; X40).*

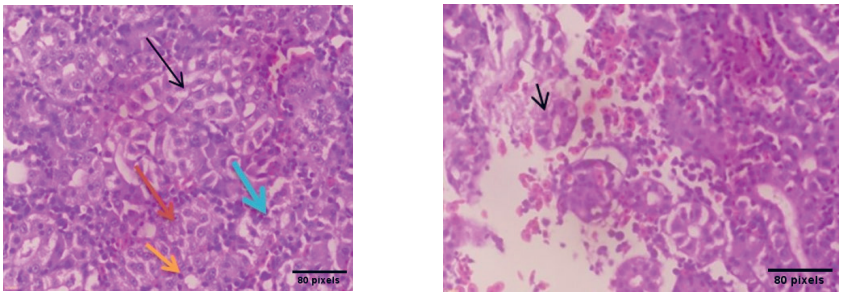

Figure 10. *Histological changes observed in kidney tissue after 3rd day (A) and 14th day (B) of ammonia exposure. (A) Cytoplasmic vaculation, pyknosis (yellow arrow) and karyorrhexis (blue arrow), karyolysis (red arrow) (H&E; X40). (B) Degeneration of the epithelium of the renal tubule (arrow) (H&E; X40).*

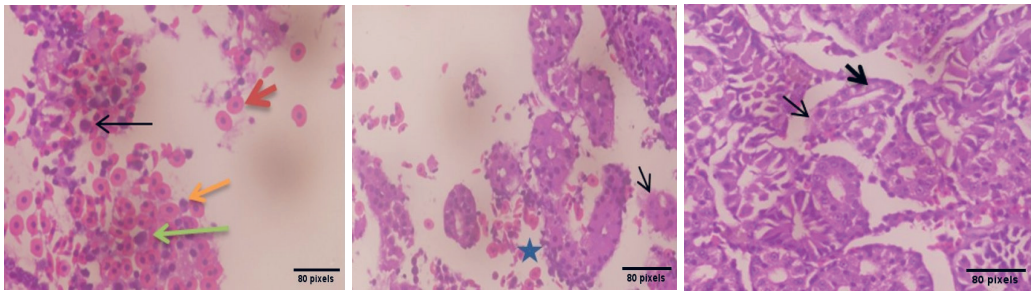

Figure 11. *Histological changes observed in kidney tissues after 3rd day (**A, B**) and 14th day (**C**) of bacterial exposure. (**A**) Lymphocyte (thin arrow), monocyte (green arrow), neutrophil (yellow arrow), immature erythrocyte (red arrow), ruptured RBC (hemolysis) (H&E; X40). (**B**) Degeneration of the epithelium of the renal tubule (thin arrow), infiltration of erythrocytes (star) (H&E; X40). (**C**) Degeneration of the renal tubule cell (thick arrow) and epithelium of the renal tubule (arrow) and (H&E; X40).*

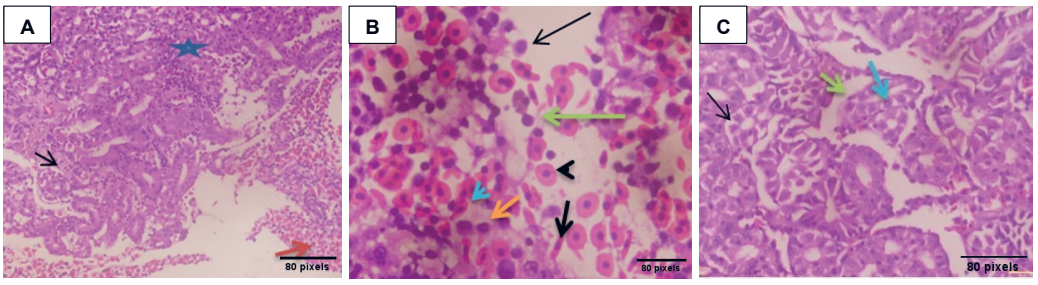

Figure 12. *Histological changes observed in kidney tissue after 3rd day (A) and 14th day (B, C) of combined exposure (both ammonia and bacteria). (A) Infiltration of erythrocyte (thin arrow), sloughing of epithelium from the basement membrane of renal tubule (red arrow), hyperplasia of hematopoietic leukocyte cells (star) (H&E; X10). (B) Large lymphocyte: irregularly round, with many projections on the surface (thinblack arrow), Small lymphocyte: elliptic, with minimal cytoplasm, and some microvilli protuberances at the margin (green arrow), Monocyte: intended shaped nucleus (yellow arrow), Immature erythrocyte (arrowhead): round, with a round nucleus, Thrombocyte: spindle-shaped with an oval and mostly centered nucleus (blue arrow), Qualitative changes in erythrocytes (elongated shape), poikilocytosis (thick black arrow) (H&E; X40). (C) Sloughing of epithelium from the basement membrane of renal tubule (thin arrow), pyknosis (green arrow) and karyorrhexis (blue arrow) (H&E; X40).*

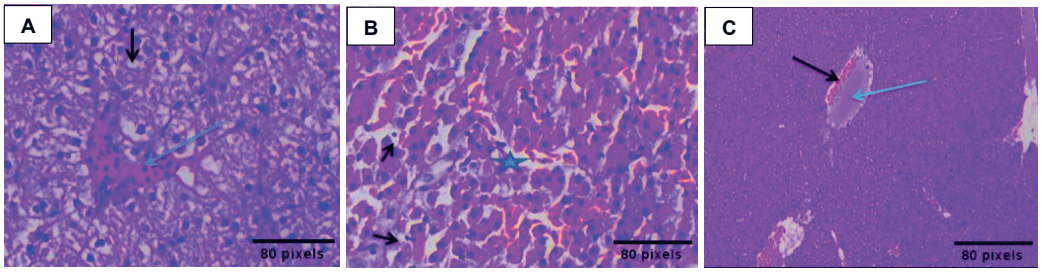

Figure 13. *Histological changes observed in liver tissue after 3rd day (A) and 14th day (B, C) of only ammonia exposure. (A) Erythrocyte infiltration into blood sinusoids (blue arrows), increased vacuolation in hepatocytes (black arrows). (B) Disarrangement of hepatic cords (star), necrosis of hepatocytes (black arrows). (C) Increased hemorrhage (black arrow), infiltration of sinusoids with erythrocyte and leukocytes (blue arrow).*

fragmentation, and hepatocyte degeneration initially, progressing to renal tubule epithelium degeneration with prolonged exposure. Bacterial infection alone leads to heavy inflammatory cell infiltration, immature erythrocyte formation, and tubular degeneration over time. Combined exposure induces erythrocyte infiltration, leukocyte

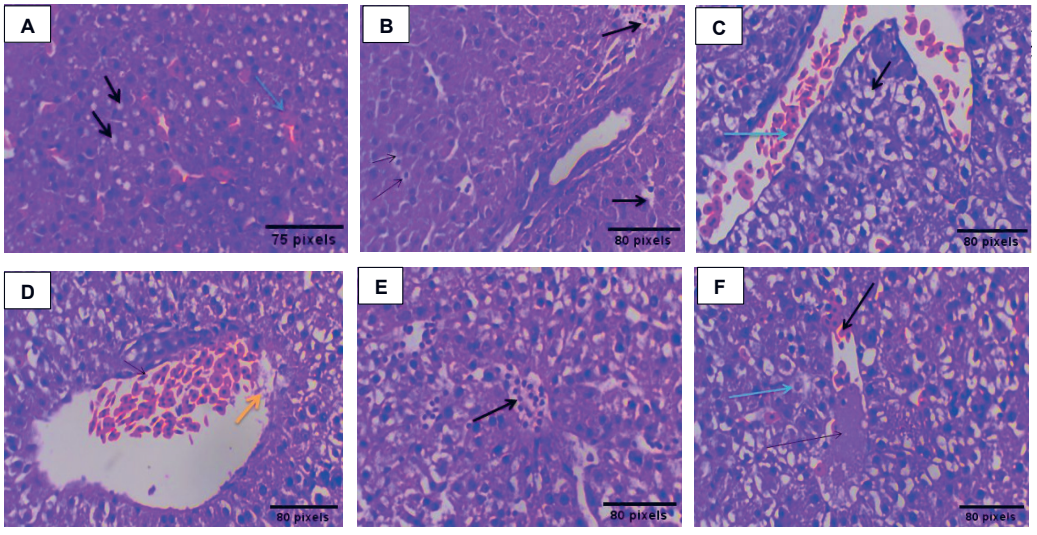

Figure 14. *Histological changes observed in liver tissue after 3rd day (A, B, C) and 14th day (D, E, F) of only bacterial exposure. (A) Increased vacuolation in hepatocytes (black arrows), erythrocyte infiltration into blood sinusoids (blue arrow). (B) Pyknotic nuclei (thin arrow), vaculation and necrosis of hepatocytes (thick black arrow). (C) Increased vacuolation in hepatocytes (black arrows), erythrocyte infiltration (blue arrow). (D) Degeneration of connective tissue and serous epithelium (yellow arrow), erythrocyte infiltration (black arrow). (E). Dilatation and congestion in sinusoids (arrow heads). (F) Hemorrhage (thin arrow), erythrocyte infiltration (thick arrow), vacuolar degeneration of hepatocytes (blue arrow).*

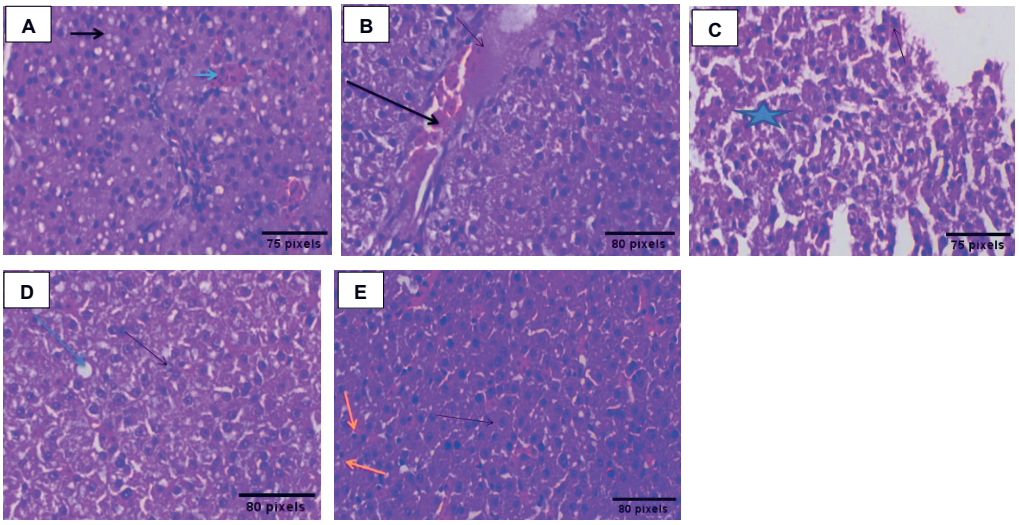

Figure 15. *Histological changes observed in liver tissue after 3rd day (A, B, C) and 14th day (D, E) of combined exposure (both ammonia and bacteria). (A) Increased vacuolation in hepatocytes (black arrows), erythrocyte infiltration (blue arrow). (B) Hemorrhage (thin arrow), erythrocyte infiltration (thick arrow). (C) Disarrangement of hepatic cords (star), nucleated hepatocyte (thin arrow). (D) Increased vacuolation in hepatocytes (blue arrows), vacuolar degeneration (thin arrow) (E) Hepatocytes have nuclei with prominent nucleoli (black arrow), condensed / pyknotic nucleus (yellow arrow).*

hyperplasia, and epithelial sloughing initially, with further degenerative changes in response to prolonged stress.

In Figures 13A–C, the liver tissues of fish exposed solely to ammonia exhibit immediate exposure-induced increased vacuolation in hepatocytes and erythrocyte infiltration into blood sinusoids. Prolonged exposure results in hepatic cord disorganization, hepatocyte necrosis, and heightened hemorrhage in the liver tissues. Figures 14A–E illustrates histological changes in liver tissue caused by bacterial infection alone. Brief exposure shows pyknotic nuclei, vacuolation, hepatocyte necrosis, heightened vacuolation, and erythrocyte infiltration. Extended exposure results in connective tissue and serous epithelium degeneration, along with liver sinusoids dilation and congestion. In Figures 15A–E, histological changes due to the combined exposure of ammonia and bacteria in *C. magur* liver tissues are depicted. Combined exposure leads to increased vacuolation, erythrocyte infiltration, hepatic cord disarrangement, and hemorrhage in the short term. Prolonged exposure results in vascular degeneration in hepatocytes and other effects in kidney tissue.

DISCUSSION

LC_{50} value of unionized ammonia (NH_3-N)

In this study, the 96-hr median lethal concentration (LC_{50}) of Unionized ammonia(UIA) in *C. magur* was found to be 7.2 mgL^{-1}, which is quite higher than the values reported for other fish species like Curimbata juveniles (0.62 mgL^{-1}) (31), GIFT Tilapia (0.98 mgL^{-1}) (32), shortnose sturgeon (0.58 mgL^{-1}) (33), juvenile burbot (0.58 mgL^{-1}) *(34)* and zebrafish (2.07 mg L^{-1}) *(35)*. However, it is comparable to the 48-hr LC_{50} of UIA in *Oreochromis niloticus* fingerling (7.4 mgL^{-1}) *(36)*, hybrid tilapia species (6.6 mgL^{-1}) *(37)* and double of the 96-hr LC_{50} value of UIA in Tra catfish (3.52 mgL^{-1}) *(38)* exposed to ammonia. The variation in LC_{50} values is attributed to the factors such as fish species, size, weight, sex, and biological behavior *(39)*. In this case the higher LC_{50} value of *C. magur* is linked to the larger size of the fish, as larger fish tend to be less sensitive to ammonia compared to younger ones *(36, 37)*.

Hematological parameters

The presence of harmful substances in aquatic environments can influence fish blood characteristics, including TEC, TLC, Hb, PCV, MCH, MCHC and MCV, serving as indicators of prevailing health and stress condition. In the present study on sublethal ammonia toxicity, a significant decrease in erythrocyte count suggests potential anemia in fish *(40, 41)*. Similarly, in fish groups exposed to bacteria, a significant decline in erythrocyte count was observed, may be due to impaired hemopoiesis *(42)* and anemia with subsequent erythroblastosis *(43)*. The decrease in total erythrocyte count observed could also be attributed to a hindered ability of the kidney to generate red blood cells *(44)*, or it could be linked to blood dilution due to disruptions in osmoregulation across the gill membrane *(45)*. A significant decline in total leucocyte count (TLC) in ammonia-exposed fish indicates a weakened general immune response in fish, as leukocytes play a crucial role in immune defense *(46)*. This decline in count could likely be linked to an increase in reactive oxygen species (ROS) resulting from prolonged ammonia toxicity *(47)*. Conversely, both bacteria and combined exposed

treatment groups initially showed an increase in TLC. The initial increment in total leucocyte count may be the indication that fish immune systems have been activated *(48)*. Although at later stage due to prolong exposure it got reduced as immunity got compromised *(49)*. A gradual decrease in Hb content in ammonia-exposed fish may be linked to oxygen deficiency resulting from weakened hematopoietic function *(50–52)* and in bacteria and combined-exposed fish may be due to impaired hemopoiesis *(42)*. Similarly, in case of hematocrit value also there was a consistent decrease in hematocrit values, akin to findings in ammonia-exposed Pacu fish *(53)*, Atlantic salmon *(54)*, and sheat fish infected with *Edwardsiella tarda (55)* suggesting potential hematopoietic tissue exhaustion or damage (56). MCV, MCH, and MCHC values also declined steadily until the 14th day across all experimental groups indicating a possible increase in immature red blood cells and a decrease in total erythrocyte counts *(57)*, similarly noted in studies on *S. schlegelii (58)* and *O. belangeri (59)*. This reduction could indicate either hemodilution or deficiencies in hemoglobin production *(60)*, consistent with observations in *A. hydrophila*-infected *Labeo rohita (61)*.

Biochemical parameters

In our study, the elevation in blood glucose level in all the experimental groups is likely due to stress-induced conversion of liver-stored glycogen into glucose, meeting the heightened energy demand caused by increased cellular metabolism triggered by stress *(62)*. In our experiment a significant decline in serum protein levels consistent with the findings in fish exposed to other toxic substances *(63)*, is attributed to the nephrotoxic effects of total ammonia nitrogen (TAN), leading to substantial protein loss through renal excretion *(64)*. This initial rise in serum protein level in bacteria & combined exposed group may stem from the release of cell contents from destroyed red blood cells *(65)*. The significant decrease in Albumin (a vital protein essential for immune function) content in our experimental groups is similar to the finding in infected Atlantic salmon *(66)*, *Cyprinus carpio (67)* and heavy metal exposed *Channa punctatus (68)*. The decrease in albumin content is likely linked to pathogen-induced hemolysis *(69)*. In all our experimental groups the initial rise in globulin content indicated an active humoral immune response in infected fish. Nonetheless, the subsequent significant reduction on the later stage may be due to prolonged stress compromising immunity *(70)*. The impact of toxic substances like ammonia on liver and kidney tissue can be evaluated by measuring SGPT and SGOT levels in blood serum *(71)*. In this study, significant increases in SGPT and SGOT levels were observed in all treatment groups (ammonia, bacteria, and combined exposure) across the sampling periods (3rd, 7th, and 14th days). Similar increase in SGOT and SGT were reported in rockfish and turbot exposed to ammonia *(58, 72)*, as well as in rainbow trout *(73)*. These elevations are probably due to tissue necrosis and increased transaminase activity *(74)*. Moreover, significant SGPT and SGOT increases were noted in rohu fish infected with *Acinetobacter pittii (75)*, consistent with findings in other bacterial infections like *Vibrio harveyi (76)* and *Aeromonas hydrophila (77)*. In this study, ammonia and combined treatment of fish showed increased urea levels in both serum and liver tissue, with the highest accumulation on day 14. Although bacteria-infected fish group showed no significant change due to low ammonia levels in the water. The ammonia-exposed fish group had a 101% increase in serum urea and a 6.5-fold rise in liver tissue. Similar findings were observed by Chew and Ip, *(78)* in African lungfish, due to detoxification of ammonia into urea. The rise in urea levels may be due to ineffective ammonia excretion in the presence of environmental ammonia, leading to detoxification via the Ornithine-urea cycle (OUC) *(79)*. In some previous studies *(80, 81)*, it has been found that magur catfish have developed several adaptive mechanisms to cope with HEA exposure, such as allowing greater ammonia buildup in various tissues, converting some of the accumulated ammonia into non-essential amino acids, and transforming it into urea through the activation of key OUC enzymes. Similarly, Banerjee *et al. (82)* have also observed that hyper-ammonia stress triggered the expression of various OUC genes. This suggests that catfish like magur has higher ammonia tolerance compared to other fish species. However, despite its ability to convert ammonia into urea, their physiology is still getting affected in the first few days of exposure, indicating the OUC cycle takes some time to efficiently excrete the excess ammonia as urea from the body though further research needs to be done to confirm the underlying mechanism.

Histopathological changes in gill, kidney and liver tissue

In our study, significant changes in gill tissues were evident. In consistent with our findings Peyghan and Takamy *(83)* also observed lamellar deformities and edema in gill tissues of sublethal ammonia exposed fish group, while Pane *et al. (84)* noted acute edema in response to heavy metals. Our findings suggest that gill hyperplasia results from increased epithelial thickness, and lamellar fusion occurs due to gill epithelium thickening via cell proliferation *(85)*. The observed histopathological changes, including partial fusion of secondary lamellae, hyperplasia, and epithelial lifting, serve as defensive responses in exposed fish, preventing toxicant entry by increasing the diffusion distance between the external environment and blood, thereby disrupting oxygen intake *(86)*. Grizzle and Kiryu *(87)* documented similar histopathological changes, including hyperplasia, hemorrhage, congestion, and necrosis in goldfish gills infected with *A. hydrophila*. Likewise, Maftuch *et al. (88)* also observed gill damage in *Myxobolus sp.* infected Koi carp (*Cyprinus carpio*) characterized by congestion and blood accumulation leading to swelling. The redness observed in our study may be due to reduced venous blood

flow, leading to increased blood volume in vessels within the gill lamellae *(89)*.

The teleost kidney plays a vital role in eliminating toxic substances and is the first organ to be affected by toxicants *(90)*. The histological findings of our study were similar with the findings of Das and Mukherjee *(91)* who had observed necrotic changes, including karyorrhexis and karyolysis, in rohu fish treated with hexachloro cyclohexane. Vacuolation and degeneration in the tubular epithelium were also observed after exposure to ammonia-N *(92)*. In the current study, a decline in mature erythrocyte count, altered shape, and increased lymphocyte populations suggested hematopoiesis failure *(91)*. At the same time, an increase in immature red blood cells in the peripheral blood suggested a compensatory erythropoietic response *(44)*. Akin to our findings, Cengiz *(93)* also documented intracytoplasmic vacuoles, pyknotic nuclei in hematopoietic tissue, and degeneration of renal tubule epithelial cells in fish exposed to toxicants. Likewise, Gill *et al. (94)* documented degeneration of tubular epithelium and nuclear damage, including karyorrhexis and karyolysis, in cadmium-exposed Puntius conchonius.

The liver organ, which plays a key role in numerous metabolic functions, is highly susceptible to the toxic effects of chemicals. Ammonia, entering the liver through the hepatic portal vein, participates in metabolic pathways *(95)*. Exposure to ammonia results in liver glycogen vacuolation, disrupting energy production and causing degenerative changes like hydropic degeneration, cloudy swelling, vacuolization, and focal necrosis in fish liver tissue *(96)*. Similar to our findings, histopathological alterations in liver of *Aeromonas hydrophila* infected *Labeo rohita* lead to congested portal vessel rupture, pyknosis, mild necrosis, and hepatocyte vacuolation. Additionally, hepatocyte vacuolation attributed to blood cell release was noted *(97)*. In consistent with our findings, *A. hydrophila infected Oreochromis niloticus* exhibited significant blood vessels and sinusoids congestion, perivascular mononuclear leukocytic infiltrations (lymphocytes, macrophages, and plasma cells), endothelial lining injury leading to perivascular hemorrhage, and thrombosis in the portal blood vessels. The hepatic parenchyma showed scattered hemorrhage, hepatocyte vacuolation, bile duct lining proliferation with periductal fibrosis, coagulative necrosis with pyknotic nuclei, tissue architecture retention, pancreatic acinar cell necrosis, and peripancreatic leukocytic infiltrations *(98)*.

Relative percentage survival (RPS)

In our current investigation, the fish exposed to bacteria exhibited the lowest survival rates, followed by the combined (ammonia and bacteria) and ammonia-exposed groups, in comparison to the control. In the present study, the combined exposure group showed relatively higher survival than the bacteria-exposed group may due to the reason as mentioned by Farmer *et al. (17)* in columnaris infected channel catfish where higher Total Ammonia Nitrogen (TAN) concentrations led were linked to less severe infections and increased survival rates compared to lower TAN concentrations. This could be attributed to the potential improvement in survivability due to ammonia exposure. The findings suggest that elevated ammonia concentrations may either hinder bacteria survival or disrupt the infection process *(17)*. Morris *et al. (99)* similarly found that increased levels of unionized ammonia in the water led to significantly improved survival rates when lost River suckers were exposed to *Flavobacterium columnare*.

CONCLUSION

The overall findings of the present study revealed that the hemato-biochemical and histopathological parameters were affected significantly higher in the case of only bacteria-exposed fish as compared to the combined exposed fish and only ammonia exposed fish group. Furthermore, the fish group exposed exclusively to bacteria displayed the lowest rates of survival, followed by the groups exposed to both ammonia and bacteria, as well as the group exposed solely to ammonia, when compared to the control. Pronounced histopathological alterations were also observed in the treatment group exposed to only to bacteria. However, urea content was higher in the ammonia exposed fish group, indicating ammonia was being converted into urea. So, from this study we can conclude that there was a negative synergistic effect or antagonism effect in the fish after exposure to ammonia and bacteria in combination. This discrepancy indicates that elevated ammonia concentrations might have restricted the survival of *A. caviae* or disrupted the infection process. It's evident that ammonia has the potential to impede the growth and survival of *A. caviae*. The findings of present study open a new avenue for further understanding of the mechanism of ammonia exposure in the direction of an alternative method to combat bacterial infections.

Acknowledgements: *The authors are thankful to the Director ICAR-CIFE Mumbai for providing all the facility and funding for conducting the research.*

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